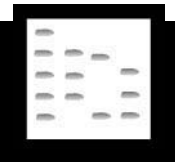
IMPREGNATION TECHNIQUES: THIN-LAYER (PLANAR) CHROMATOGRAPHY



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Introduction

The use of impregnated phases in thin-layer chromatography (TLC) has a long history, beginning in the very earliest days of the technique. As a consequence, much of the key literature in this field dates back to the 1960s and 1970s. The literature also contains a considerable number of examples of impregnation reagents where subsequently the methods have received little attention except in reviews of the subject.

The impregnation of the stationary phase in thin-layer, or planar, chromatography can be used to achieve a number of ends. These include changing the mode or selectivity of the chromatographic system, improving chromatographic performance and enhancing detectability. An example of the use of impregnation to change the type of chromatography would be the use of a nonpolar, water-immiscible material such as paraffin oil which can be used to enable reversed-phase separations to be performed on silica-gel TLC plates. Alternatively, plates impregnated with silver nitrate can be used to improve the separation of compounds containing double bonds (argentation chromatography). Similarly, boric acids or boronates are useful for compounds such as carbohydrates and sugars containing vicinal diols, and ion pair reagents provide a means for successfully separating polar ionic species. Although the use of impregnation techniques for enhancing detectability will not be considered in depth here, examples would include the incorporation of ammonium acetate followed by heating, which can result in the formation of intensely fluorescent derivatives, whilst paraffin oil impregnation can stabilize or enhance fluorescence and the radiolabelled substances can be detected using impregnation with a scintillant.

There are a variety of methods for obtaining impregnated layers, including the preparation of the TLC plate with slurries containing both the stationary phase and the impregnating reagent, pre- or post-chromatographic dipping, pre- or post-chromatographic development of the plate in a solvent containing the impregnating agent or spraying, and these are described below.

Impregnation Techniques

All of the techniques for impregnating TLC plates described below have been used at one time or another and are illustrated with examples in the subsequent sections on applications.

Spraying

Spraying the TLC plate is a simple and convenient method for applying the reagents used for detection in TLC and indeed is widely used for this type of application. It is, however, much less well suited to the impregnation of TLC plates in order to modify chromatography because of the difficulty of ensuring uniform coverage. It also requires much skill and expertise in order to ensure reproducible results from day to day. Both of these technical difficulties are clearly disadvantages, and spraying is therefore not recommended for routine applications in this area. Spraying is therefore probably best used, if at all, for one-off applications or for screening phases and impregnating reagents for a particular property during method development.

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Dipping

Dipping plates in solutions of the impregnating reagent in a suitable (usually volatile) solvent is probably the simplest and most efficient method of ensuring even coverage of the stationary phase with the reagent of choice. Suitable dipping chambers, of the type used for immersing plates in solutions of chromogenic visualization reagents, are available from a number of manufacturers at modest cost. These chambers have relatively small volumes and so are not expensive in reagent. Some automated devices are also available that can be used to control the impregnation time accurately. Dipping, in general, because of the ease of control over parameters such as reagent concentration and impregnation time, should be considered to be the preferred method for impregnating TLC and HPTLC plates.

Pre-development

It is also a relatively easy matter to prepare TLC plates impregnated with the desired reagent by pre-development in a solution of reagent in a suitable solvent. This technique is also referred to as irrigation of the plates. The solvent used for this purpose needs to be sufficiently eluotropic to ensure that any

affinity of the reagent for the stationary phase is overcome or else, at worst, the plate will not be impregnated with the reagent which will stay at or near the origin (or else be distributed as a gradient of decreasing concentration up the plate). Nonmigration of the impregnating reagent is seen with, for example, the ion pair reagent cetrimide if dissolved in water and then used to coat reversed-phase TLC plates in the pre-development method. Because the reagent is relatively nonpolar it has a strong affinity for the stationary phase and remains at the origin. In such cases dipping is to be preferred.

Mixed Phases

Although with the advent of good quality commercial TLC and high performance TLC (HPTLC) products the preparation of TLC plates in the laboratory is no longer widely practised, one method of producing impregnated plates was to produce mixed phases. Thus, the bulk stationary phase (generally silica gel) was mixed with an appropriate amount of the impregnating reagent, a binding agent, slurried in a suitable solvent and spread as a layer on glass plates. After drying and activating (if necessary), the plates were then used as required. Some manufacturers provide a range of mixed phases (e.g. silver nitrate-impregnated plates, buffered layers, ammonium acetate-impregnated layers, etc.).

Specific Examples of Impregnation in TLC

Impregnation to Form Lipophilic Stationary Phases for Reversed-phase Separations

Before the introduction of good-quality bonded phases, the preparation of suitably hydrophobic stationary phases used to be a very popular method for obtaining layers with which to perform reversed-phase separations, and still has many advantages. This is readily achieved using liquid paraffin oil, undecane, *n*-decane, nitromethane, propylene glycol, silicone oil, decalin, Carbowax 400, formamide, 2-phenoxy- and 2-methoxyethanol, various mineral oils, and substances such as ethyl oleate and similar materials. In addition to modifying chromatography, impregnation with these materials can also be used to enhance or stabilize the fluorescence of suitable analytes, thus improving detection and quantification. However, it is the use of these materials to provide reversed-phase separations that is of interest here.

A typical example of the use of paraffin oil is provided by work on the reversed-phase separation of ecdysteroids (polar, polyhydroxylated steroids found in plants and arthropods). Here silica-gel TLC and HPTLC plates were impregnated with a solution of 7.5% paraffin oil in dicholoromethane (v/v) by dipping. The plates were air-dried and chromatography was performed using methanol–water mixtures. In an interesting variation on this general technique of impregnation, a variety of normal-phase separations, for nonsteroidal anti-inflammatory drugs, ecdysteroids, antipyrine and aminophenols, on silica gel were performed with the paraffin added to the mobile phase. Addition of 7.5% (v/v) of paraffin to the normal-phase solvent system did not affect chromatography but did enable the plate to be impregnated so that a subsequent reversed-phase separation in a second dimension could be undertaken immediately that the solvent from the normal-phase separation had evaporated. An example of this for the nonsteroidal anti-inflammatory compounds is shown in **Figure 1**. It should be noted that, with this type of impregnated plate it is also possible to perform the reversed-phase separation first, remove the nonpolar impregnating reagent with a suitable solvent (i.e. one that does not affect the analytes but does remove the impregnating reagent) and then perform a normal-phase separation in the second dimension. Alternatively, a similar outcome can be achieved by impregnation of that portion of the plate not used for

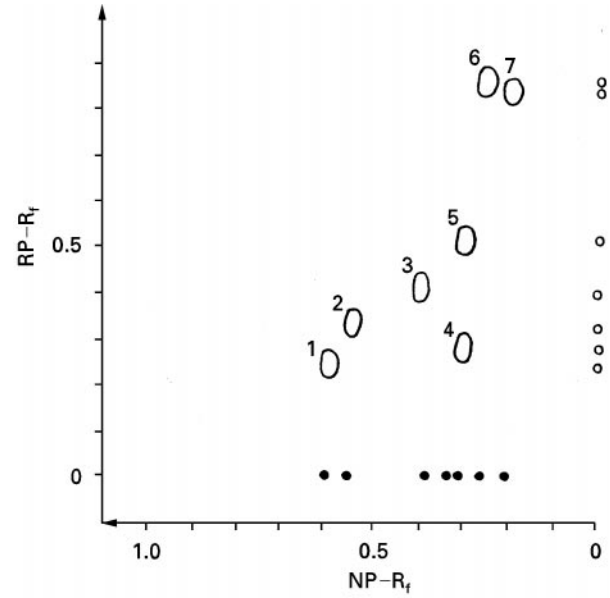

Figure 1 Two-dimensional separation of nonsteroidal drugs with paraffin impregnation during normal-phase (NP) separation in the first dimension to enable reversed-phase (RP)-TLC to be carned out in the second. 1, Ibuprofen; 2, ibufenac; 3, methyl analogue of isoxepac; 4, indomethacin; 5, isoxepac; 6, salicylic acid; 7, 5-methoxysalicylic acid. Details in Wilson ID (1984) Normal-phase thin-layer chromatography on silica gel with simultaneous paraffin impregnation for subsequent reversed-phase thin-layer chromatography in a second dimension. *Journal of Chromatography* 287: 183–188.

the separation in the first dimension, and examples of a normal-phase separation followed by impregnation with 2-phenoxyethanol or undecane for reversed-phase chromatography in the second dimension have been described.

Impregnation with Silver Nitrate

Argentation TLC also represents an important methodology and is considered in detail elsewhere in this work and so will only be briefly described here. The impregnation of TLC plates with silver nitrate has been used for many years as a means of improving the separation of unsaturated compounds, particularly certain lipids, based on the ability of silver ions to form charge transfer complexes with the π electrons of the carbon–carbon double bonds. In general, silica gel is used: the amount of silver nitrate used varies from as little as 2% up to 20 or 30% w/w depending upon the author and application. With silica gel, impregnation by both spraying (with a 10–20% solution in either water or methanol) and the preparation of mixed phases have been described. Following preparation it seems to be good practice either to use the plates the same day or else to store them in a sealed container in the dark until required. In general, non-polar solvent systems are employed (e.g. pentane, hexane-diethyl ether, chloroform-methanol, diethyl ether, light petroleum, etc.).

Whilst the use of silver nitrate-impregnated plates has generally been with normal-phase separations on silica gel, there has been recent work using reversed-phase (C_8-bonded) layers dipped in solutions containing between 0.5 and 4% silver nitrate for 10 s. These plates were investigated for their ability to separate the *cis*/*trans* isomers of capsaicin, and comparison was made simply using the silver nitrate in the mobile phase (60 : 40 methanol–water v/v). With impregnation no effects were observed until the 2% (w/v) impregnating solvent was used, and even with 4% this was insufficient to provide the required resolution. In contrast, when present as a mobile-phase additive, even as low a concentration as 0.5% w/v was sufficient to give baseline resolution. It appears likely from this result that the high solubility of the silver nitrate in the mobile phase led to its rapid elution from the impregnated plate, with consequent loss of effect. Thus, interestingly, it seems from this work that the use of the silver nitrate in reversed-phase systems is only practicable when it is present as a mobile-phase additive.

Impregnation with Polyol and Sugar Complexing Reagents

The ability of borate ions to complex with suitable polyhydroxylated compounds such as carbohydrates is well known and has provided the basis of a number of methods for their separation. TLC with layers impregnated with sodium arsenite, phosphotungstic, tungstoarsenate and molybdic acids have also been shown to have useful properties for the resolution of mixtures of oligo and monosaccharides.

Various methods have been used to prepare such layers, including both spraying and the preparation of mixed layers. Thus, in an early example of the use of boric acid, sodium borate and sodium arsenite plates were either sprayed with methanolic or aqueous solutions containing 10–20% of the impregnating reagent or plates were prepared by mixing 2.8 g of the reagent in 50 mL of water with 25 g of silica gel G to give a 10% (w/w) mixed layer. These layers were then used to separate the *erythro* and *threo* isomers of a variety of di- and trihydroxy long chain fatty acid esters.

Similar work on phosphotungstic and molybdic acid impregnated silica gel TLC plates showed them to be particularly useful for the separation of oligosaccharides giving complexes with higher R_f values than the corresponding boric acid complexes under similar conditions. In this case the plates were made by mixing 35 g of the chromatographic stationary phase (e.g. silica gel–alumina 1 : 1 or 3 : 1 or alumina) with 70 mL of an aqueous solution containing an appropriate amount of the impregnating reagent and spreading the plates as a 0.4 mm layer on to glass. The resulting plates were then dried at room temperature for 24 h, and heated for 1 h at 110°C before use. In general, the best results were obtained with TLC plates treated with phosphoric acid–sodium tungstate or saturated molybdic acid as impregnating reagents.

Impregnation with Liquid Ion Exchangers and Neutral Organophosphorous Compounds for Metal Ion Separations

The separation of inorganic ions has been an important application of TLC. Another area that has proved to be of some interest for the use of the impregnation technique as a means of improving analyte resolution in TLC has been the employment of the so-called liquid ion exchangers. This developed from the widespread use of this type of reagent in paper and column chromatography. A range of these liquid ion exchangers were used in early examples of this type of application. However, extensive experimentation suggested that adogen 464, alamine 336, amberlite LA1 and primene JM-T were suitable for this type of application. In these early studies a 0.1 mol L^{-1} solution in chloroform was used for impregnation of the silica gel, with plates prepared from a suspension of the silica gel in this solution. Metal ions on TLC have also been resolved on layers

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impregnated with neutral organophosphorous compounds such as tri-*n*-butyl phosphate and tri-*n*-octylphosphine oxide. The bulk of the early literature in this area was collated by Brinkman *et al*. (see Further Reading).

More recent examples of the use of impregnation for metal ion separations have included further examples of the use of primene JM-T, amberlite LA-1 and LA-2, alamine 336 and aliquat 336, tri-*n*-octylamine, tri-*n*-butyl phosphate and tri-*n*-butyl amine-impregnated silica gel TLC plates.

Impregnation with Ion Pair Reagents

The use of ion pair reagents is also discussed in detail elsewhere in this work and will therefore only be briefly described here. A number of workers have shown that ion pair reagents can be used as mobile-phase additives or following impregnation in the stationary phase for the subsequent chromatography of polar organic compounds. The methodology used depends to a large extent on the nature of the reagent, but also to some degree on the stationary phase. So, whilst both silica gel and alkyl-bonded layers can be treated with these reagents, the results are generally better with the bonded phases. In addition, it should be noted that low molecular mass ion pair reagents such as tetramethylammonium salts are so soluble that simply impregnating the stationary phase is generally ineffective as, with aqueous solvents, the reagent rapidly dissolves in the mobile phase when chromatography is initiated. This rapidly depletes the amount of reagent available for ion-pairing and results in a generally unsatisfactory chromatographic result. Other reagents, however, typically long chain sulfonic acids or quaternary ammonium compounds (e.g. sodium dodecyl sulfate or cetrimide) are only effective when the plate has been impregnated with the reagent (as a solution in a volatile organic solvent such as chloroform or ethanol) prior to chromatography. We have found that dipping is the most effective means of ensuring an even coating of the layer with these substances.

Impregnation with Chiral Selectors

Chiral separations in TLC have been accomplished using chiral stationary phases, chiral mobile-phase additives and by impregnating suitable TLC phases with chiral selectors. Probably the best characterized separation of this type is based on chiral ligand exchange where the chiral selector (2*S*, 4*R*, 2′*RS*)-4-hydroxy-1-(2′-hydroxydodecyl)proline/copper [II] acetate impregnated into C_{18}-bonded TLC or HPTLC plates. These plates are excellent for the separation of chiral amino acids and related compounds, and substances such as α-hydroxycarboxylic acids. Plates of this type are commercially available from several manufacturers (as the CHIR and Chiral Plate from Merck and Macherey Nagel, respectively). A typical series of separations on the CHIR HPTLC plate is shown in **Figure 2**. Alternative systems based on a similar mechanism involve the use of *N*,*N*-di-*n*-propyl-*L*-alanine or poly-1-phenylalaninamide copper complexes.

Another example of the impregnation approach to the chiral TLC separations involves the impregnation of diol-bonded HPTLC plates with the chiral ion pair reagent *N*-benzoxycarbonyl-glycyl-L-proline (ZGP). These plates are prepared by first washing with dichloromethane, then dipping in a 4 mmol L^{-1} solution of ZGP and 0.4 mmol L^{-1} ethanolamine. Following drying these plates can be used to separate the enantiomers of β-blockers such as popranolol using chloroform–ethanol solvent systems.

It is also possible to prepare a Pirkle-type stationary phase for chiral separations by dipping aminopropyl-bonded silicia gel HPTLC plates into solutions of substances such as (*R*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine or (L)-*N*-(3,5-dinitrobenzoyl)leucine

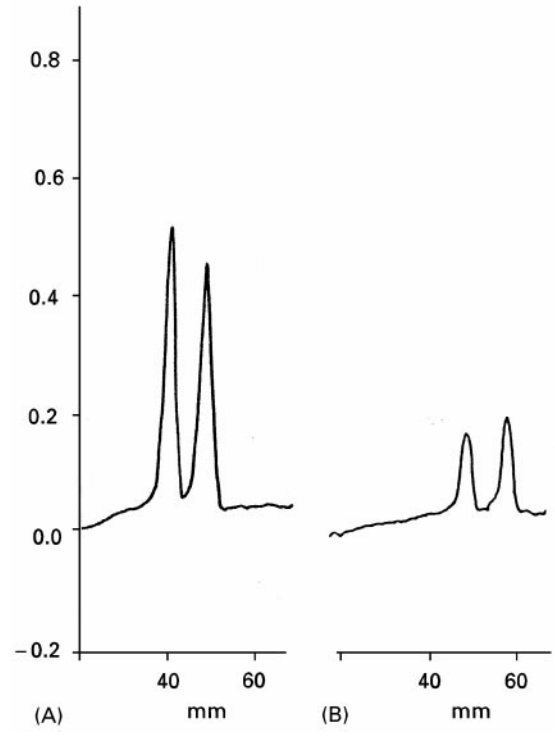

Figure 2 Separation of the enantiomers of (A) isoleucine and (B) leucine on the CHIR HPTLC plate (detection using ninhydrin) with a water–methanol–acetonitrile mobile phase (50 : 50 : 200 v/v). Details in Wilson ID, Spurway TD, Witherow L, Ruane RJ and Longden K (1990) Chiral separations by thin-layer chromatography. *Recent Advances in Chiral Separations*, pp. 159–168. New York: Plenum.

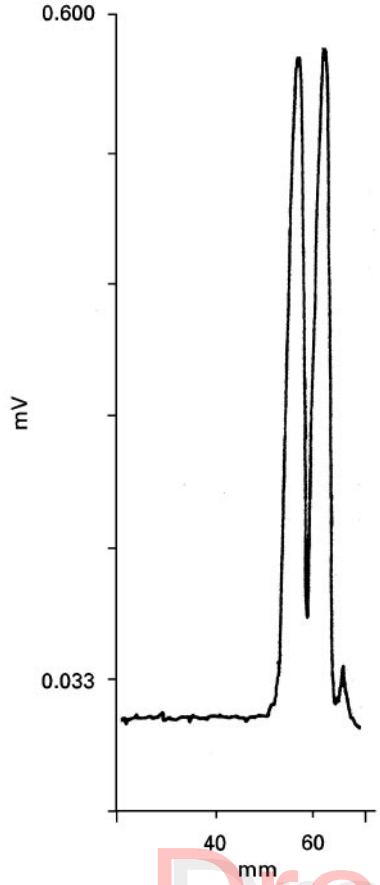

Figure 3 Separation of the enantiomers of 2,2,2-trifluoro-(9-anthryl)ethanol on aminopropyl bonded HPTLC plates modified by impregnation with *N*-(3,5-dinitrobenzoyl)-L-leucine to form an ionically bonded Pirkle-phase. Details in Witherow L, Spurway TD, Ruane RJ and Wilson ID (1991) Problems and solutions in chiral thin-layer chromatography: a two-phase 'Pirkle' modified amino-bonded plate. *Journal of Chromatography* 553: 479–501.

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(0.5 mol L^{-1}). Since what is being formed is essentially an ionically bonded stationary phase, it is arguable that the plate is not being impregnated. However, the process that is performed, and the overall effects, are indistinguishable from conventional impregnation and the methodology is included for completeness. An example of a typical separation on such a modified plate is shown in **Figure 3**.

Impregnation with Oxalic Acid

The modification of TLC plates with oxalic acid has been performed to good effect for a range of solutes, including fatty acids, insecticides, nonionic detergents, azulenes and amines. In the example of the azulenes, the TLC plates were prepared by spreading the silica gel in a aqueous solution of 0.19 mol L^{-1} oxalic acid, as immersion was found to give less satisfactory results. The plates were then activated by allowing them to dry for several hours over desiccant silica gel (activation using heating in an oven gave similar but irreproducible results). This activation was quite critical for the achievement of a good separation, as plates that were too damp gave no resolution. For the TLC of certain acidic mycotoxins, the use of silica gel TLC plates that had been immersed in a 10% solution of oxalic acid in methanol for 2 min and then activated by heating at 110°C for 2 min enabled tailing to be eliminated and resulted in well-defined spots.

Impregnation with Inorganic Buffer Salts

The use of phases impregnated with sodium or potassium salts has been described for a number of solutes. Thus, conjugated dihydroxy bile acids were separated on silica gel plates that had been impregnated by dipping in a solution of potassium dihydrogen phosphate. However, a major application of this type of impregnation has been for carbohydrates. As examples, glucose, fructose and sucrose in molasses were resolved on silica gel HPTLC plates dipped in 0.2 mol L^{-1} aqueous solution of monobasic potassium phosphate. In contrast, when this separation was attempted on nonimpregnated plates it failed. Similar results have been observed by other workers with this type of sample and in addition to potassium-based buffers, plates buffered with 0.15 mol L^{-1} sodium dihydrogen phosphate (prepared by mixing Kieselghur with the buffer prior to spreading the plates) have also been used to separate sugars (fucose, xylose, ribose, etc). Other workers have also examined impregnation of silica gel, prepared by spreading the adsorbent in a solution of the appropriate salt, with a range of sodium salts (phosphates, acetate, sulfate, phenyl phosphate) for a wide range of carbohydrates, including mono- and oligosaccharides and uronic acids. From these studies the best separations of monosaccharides and uronic acids were obtained with plates impregnated with 0.2–0.3 mol L^{-1} salt concentrations, whilst oligosaccharides gave the best results with 0.05–0.1 mol L^{-1} salt solutions. Overall, from the published examples, it seems clear that the TLC separation of carbohydrates does benefit from the use of this type of impregnating reagent.

Charge Transfer Complexes for the Resolution of Polynuclear Aromatic Hydrocarbons

A range of compounds have been used to impregnate silica gel TLC plates in order to improve the

resolution of compounds such as the polynuclear aromatic compounds (fluoranthene, benzopyrine, etc.) based on the formation of charge transfer complexes with different electron acceptors. These reagents have included caffeine, tetracyanoethylene, 1,3,5-trinitrobenzene, picric acid, chloranil, bromanil, benzoquinone and similar compounds, 2,4,7-trinitrofluorenone, teramethyluric acid, urea, pyromelliticdianhydride, 9-dicyanomethylene-2,4,7-trinitrofluorenone, sodium desoxycholate, dimethylformamide, styphnic acid and various amino acids and nucleic acid bases. However, although a wide range of reagents have been impregnated into TLC plates in an attempt to enhance the separation of polynuclear aromatic hydrocarbons, not all have been equally effective: in one study picric acid was described as values, whilst styphnic acid showed some effect and trinitrofluorenone (on alumina) proved to be excellent. Some of the reagents listed above also proved to be heat- or light-sensitive further restricting their utility.

A recent example of the use of caffeine, shown by several groups to have a profound effect on the TLC of polynuclear aromatic hydrocarbons, as a means of improving the HPTLC resolution of the polynuclear aromatic hydrocarbons, by charge transfer complex formation, involved preparing a solution of 4 g of the reagent in 96 mL of dichloromethane. Silica gel plates (with a preconcentration zone) were then dipped in this solution for 4 s and dried at 110°C for 30 min. Then, before sample application, the plates were pre-washed by running a blank chromatogram with dichloromethane as the mobile phase with subsequent reactivation at 110°C and then preconditioning the impregnated plate for 30 min at 100% relative humidity prior to sample application and chromatography. Following sample application, development was performed with diisopropyl ether–*n*-hexane (4 : 1) as the solvent in an unsaturated TLC tank. Although chromatography could be performed at room temperature, the best results (especially where quantification was to be performed) were obtained when the plates were developed at 22°C. The results of this type of separation, at room temperature and − 22°C, are illustrated in **Figure 4**.

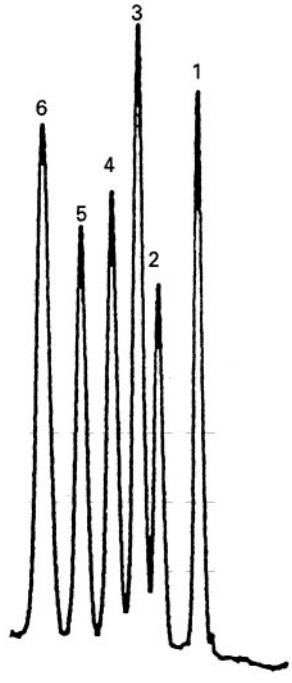

Figure 4 Fluoresence scan of a separation of polynuclear aromatic hydrocarbons (2 ng per spot, except 6 which was 10 ng per spot) on a caffeine-impregnated silica gel layer with chromatography performed at − 20°C. 1, Benzo (ghi) perylene; 2, benzo (a) pyrene; 3, benzo (b) fluoranthene; 4, benzo(k)fluoranthene; 6, fluoranthene. Reproduced with permission from Funk W, Gluck V, Schuch B and Donnevert G (1989) Polynuclear aromatic hydrocarbons (PAHs): charge transfer chromatography and fluorimetric determination. *Journal of Planar Chromatography* 2: 28–32.

An added benefit of this type of system is that some of the reagents used for charge transfer chromatography also result in enhanced detection of the compounds of interest because of the highly coloured or even fluorescent complexes that are formed with reagents such as chloranil or pyromellitic dianhydride.

Miscellaneous Impregnation Reagents

As well as the areas described above, there are a large number of other applications of impregnation to modify the properties of the stationary phase in TLC. Some of these are briefly outlined below in order to give an indication of the extent of this type of work, but the list is by no means exhaustive.

Plates impregnated with ethylenediaminetetraacetic acid have been employed for the chromatography of certain antibiotics (e.g anthracyclines and tetracyline), mycotoxins, citrinin and 8-hydroxyquinolone derivatives, and may confer benefits when the TLC of metal chelating compounds is performed.

TLC plates have also been impregnated with a variety of metal salts for particular compounds or classes of compounds. These include ferric chloride on Kieselguhr (oxine derivatives), zinc salts on silica gel and silanized silica gel (chlorinated anilines, carbamates), cadmium salts on silica gel (aromatic amines), manganese salts on silica gel (aromatic amines), copper sulfate on silica gel (hexosamines. glycosamines, barbiturates), thallium nitrate on silica gel (monoterpene hydrocarbons) and lithium

chloride-impregnated silica gel (pyrrolizidine alkaloids). Magnesium acetate has been used for phospholipids. In addition, lead salts have been employed as impregnating reagents to modify the separations of sugars and polyols. Recently, ammonium cerium (IV) nitrate was used for the separation of aromatic amines.

Other types of impregnation include the use of silica gel with phenol for aliphatic and aromatic amines following impregnation with 2% aqueous solution of the reagent. For the aromatic amines, phenol itself was found to be the most useful reagent, providing a significant improvement in spot shape was noted for a wide range of anilines compared to chromatography on the untreated stationary phase. In the case of aliphatic amines *o*-chlorophenol gave the best result. For both classes of compound this improvement in chromatography was assumed to be due to hydrogen bond formation between the reagent and the solutes. The chromatography of phenols on cellulose has also been modified by impregnation with 10% polyamide, and on silica gel with 20% polyamide. In addition, aniline-impregnated layers have been used for phenol derivatives whilst sodium nitrite-impregnated silica gel has also been shown to provide separations that could not be achieved on native silica gel. Carbonyl compounds were modified by derivatization to 2,4-dinitrophenylhydrazones on alumina TLC plates impregnated with silver nitrate (44%, w/w). Urea impregnation, described above for polynuclear aromatic hydrocarbons and related compounds, has also been used for the separation of lipid classes on silica gel plates.

Concluding Comments

As indicated in the introduction, impregnation techniques have been employed since the earliest days of TLC and their use greatly extends the versatility of TLC by enabling more selective separations or detection. To some extent the increasing availability of bonded layers has reduced the need for the preparation of silica gel layers impregnated with nonpolar materials such as paraffin oil in order to perform reversed-phase separations. However the usefulness of silver nitrate-impregnated phases for the separation of compounds containing double bonds remains undiminished, and similar observations could be made for many of the impregnation reagents described above. A continued role for impregnated stationary phases in TLC therefore seems likely.

Further Reading

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IMPRINTED POLYMERS: AFFINITY SEPARATION

See **III / SELECTIVITY OF IMPRINTED POLYMERS: AFFINITY SEPARATION**